THE EMBO JOURNAL

Manuscript EMBO-2012-81936

A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy

René Hägerling, Cathrin Pollmann, Martin Andreas, Christian Schmidt, Harri Nurmi, Ralf H. Adams, Kari Alitalo, Volker Andresen, Stefan Schulte-Merker and Friedemann Kiefer

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Review timeline:

Submission date:	03 May 2012
Editorial Decision:	23 May 2012
Additional Author Correspondence:	31 May 2012
Additional Editorial Correspondence:	01 June 2012
Revision received:	01 December 2012
Accepted:	05 December 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 May 2012

Thank you for the submission of your manuscript to The EMBO Journal. We have just now received the full set of reports from the referees, which I copy below. As all three referees think that your manuscript is interesting and their comments are quite positive, I would like to ask you to revise it according to the referees' comments.

As referee reports are quite explicit and mainly ask for clarifications, I will not repeat their arguments here. I would nevertheless like to draw your attention to the comments from referee #1, particularly to the suggestion of performing similar analyses on Prox1 and VEGFR3/VEGF-C mutant mice. After all, these factors are key players in the lymphangiogenic process. This extra information could be accommodated in the manuscript by following some of the suggestions of referee #2, although only redundant/low resolution information should be removed or moved into supplementary information.

Do not hesitate to contact me in case you have any question on how to proceed, need further input or you anticipate any potential problem in fulfilling the referee requests.

Please be aware that your revised manuscript must address the referees' concerns - particularly the ones detailed above - and their suggestions should be taken on board. Keep in mind that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will

form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process iniciative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (General Remarks):

By combining genetic approaches and optical sectioning microscopy, the authors identify two new structures of developing lymphatic system: the dorsal peripheral longitudinal lymphatic vessel and the ventral primordial thoracic duct .
The description is rigorous and the figures shown are largely informative. However the use of Cxcr4 and CCBE1 null mice are not sufficient to describe a solid mechanism explaining the formation of these structures. I suggest to extend the behavior of pTD and PLLV in Prox1 and VEGFR3 or VEGF-C null mice. Furthermore most of the data shown should be connected to and supported by morphometric analyses of the phenomena described.

To better support the concept underlined in the II paragraph of page 6 I suggest to show embryos at 9.5 and 10.5 at low magnification as in panel A and B of fig 1 with endomucin and Prox1 both associated with PECAM.

Fig 1T. How do the authors record the nuclear shape?

Page 7. "The transition was associated with a pronounced...in iLEC (...)." May the authors show that the shaped modified iLEC really increase the expression of VEGFR3, Nrp2 and Prox1? How do the authors support this claim. It is required to show an accurate morphometric analysis. Similar information have to be provided for the description of other gene expression modifications along the text.

Page 7 last paragraph. An elegant experiment to really confirm that LEC number increases from E10 to E10.25 should be done by in vivo BrdU labeling. (similar experiment have been provided for earlier stages, fig s6).

Fig 2. Which is the panel corresponding to scheme G? I suggest to show sLEC in the wholemount pictures. Alternatively, refer to fig 3C.

Fig 2B. Which the rationale for the use of Unc5B? Some of the explanation provided later in the text should be indicated here.

Fig 4E. What is the PECAM positive "ring" near CV?

In Fig 4F the authors describe the interaction between an arterial vessel and CV. This point should

be discussed and a possible speculation of this role exploited.

Fig 5I. Which is the quantitative basis of the score here reported?

Page 10, first lines. Which is the rationale supporting that valves develop from these Prox1 positive structures? I suggest examining the presence of the contact described in fig 4 in Prox1 and VEGFR3 null mice .

Fig 6. To facilitate the reader I suggest showing again the schemes of wild type mice.

Fig 7. Does Cxcr4 ablation modify PPLV condensation?

OTHER POINTS

Fig 1a,B. I suggest to indicate CV not only in the legend but also in the panels.
Fig 7. The title of figure is confusing
Fig 4B "Hear " is probably a mistake
Fig 1 , panel S. To this referee it is not clear which panel this scheme corresponds.
Fig 1N,O. There is not correspondence between the text (page 7, III paragraph, line 8) and the embryo stage indicated in the panels. E11 corresponds to Fig 5.

Referee #2 (General Remarks):

H‰gerling & Pollmann et al. present an impressive amount of beautiful images depicting early stages of lymphatic vascular development in E9.5-12 mouse embryos. In contrast to recent results of Francois et al., this works shows that lymphatic endothelial cells, arising in the cardinal vein, exit as strings of migratory spindle-shaped cells. These cells, which have higher expression of Nrp2, Prox1 and Vegfr3, then coalesce into a mesh-like network and further condense into two large vessels, a peripheral longitudinal lymphatic vessel, from which secondary more superficial structures arise, and a primordial thoracic duct, connected to the vein via a lymphaticovenous valve.

The study also describes the expression of some known lymphatic markers during the different early steps that give birth to the lymphatic vascular network.

The technique is further used to analyse Ccbe1-/- embryos, which are known not to develop definitive lymphatic structures. As reported earlier, Prox1+/Vegfr-3+ lymphatic endothelial cells are shown to emerge in the cardinal vein. A new finding of this work is that there are ectopic Prox1+ cells in ISVs and some of these cells are still able to form rudimentary lymphatic vascular structures at very early stages, which then disappear later, as these presumptive lymphatic endothelial cells die. Still, the majority of lymphatic endothelial cells seem unable to bud and remain in the venous wall before disappearing. More interestingly, in the absence of Ccbe1 expression of Vegfr-3 is not extinguished in venous endothelium, and there is aberrant sprouting from this vascular compartment.

Finally, lymphatic vasculature in Cxcr4-/- embryos is analysed. In contrast to zebrafish, inactivation of Cxcr4 in mouse does not affect early steps of lymphatic vascular development, but the dermal lymphatic vessels display an abnormal patterning at E14.5.

The manuscript contains very nice images and it reports detailed high-resolution analysis of early lymphatic vascular development. Until now this process was only studied using OPT, a technique with much lower resolution, and coronal sections (Francois et al., Dev Biol., 2011), which apparently led to somewhat erroneous conclusions. Therefore, the data of H‰gerling et al. are important because they provide a hopefully definitive morphological description of the process. However, in the current format the manuscript is too complicated, contains too many redundant images and it is rather difficult to understand. Study of the expression of some selected lymphatic vascular markers is descriptive and does not add much to better understanding of the molecular events involved. In contrast, very interesting phenotype of Ccbe1 deficient mice is described only briefly. More detailed comments are outlined below:
MAJOR REMARKS

1. The manuscript should be re-written and shortened to emphasize two essential point of the manuscript, i.e. the new model for emergence of lymphatic vasculature, and better characterization of Ccbe1 knockout mice. If such possibility exists, I would recommend a short report format.
2. The number of images should be reduced by approximately half, only essential high magnification images clearly illustrating the point should be shown. In my view, almost all figures require this clean-up. As example, in figure 1N, O only one picture, that illustrates changes in nuclear shape should be retained. In supplementary figure 8, only image per developmental stage showing the expression domain of Ccbe1 should be shown, which will make three images instead of current nine, etc.
3. The final figure should contain the comparison of the previous and new scheme that authors propose for formation of early lymphatic vasculature.
4. Description of expression of lymphatic markers should be shortened or re-written. In the present manuscript, this description looks like a patchwork, and one wonders why expression of other molecules, important for vascular development, for example Vegfr2 (spouting), ephrinb2 (Vegfr2/3 signaling), integrin alpha9 (vessel maturation and LECs migration), integrin beta1 (lumen formation) was not studied?
5. Downregulation of Vegfr-3 in blood vessels, emergence of podoplanin expression only at E11 and lack of Lyve-1 expression in early dermal lymphatic vessels are not entirely new findings, corresponding papers (Kaipainen et al., 1995, Schacht et al., 2003, Norrmen et al, 2009) should be cited. High Nrp2 and low Lyve1 in migrating LECs were also described by Francois et al., 2011.
6. Description of Ccbe1 phenotype is confusing. Do the authors suggest that in the absence of Ccbe1 there is ectopic trans-differentiation of LECs in ISVs and other venous endothelium? How does it fit with the statement that Ccbe1 is necessary for the maintenance of Prox1 expression? What is the mechanisms for increased Vegfr-3 expression? Was the expression of Nrp2, netrin-4 and Unc5b modified in Ccbe1 ko embryos, which may in part explain sprouting phenotypes? In any case, this part needs to be better structured and presented more clearly
7. CXCR4 story does not really fit with the other parts of the study, which focuses on the very early steps of lymphatic vascular development. At E14.5 in the skin the lymphatic developing vasculature is still growing as an immature primary plexus and the differentiation into capillary and collecting vessels is therefore difficult to visualize at that early stage. Given a rather superficial characterization of Cxcr4 knockout phenotype, this part can be removed without affecting main message (and perhaps published separately after more detailed analysis).

Minor remarks

1. The paragraph at the beginning of Resuts secrion describing microscopy is very technical, I suggest to shift it to the Materials and Methods.
2. Some paragraphs of the discussion are redundant with the results part and some others are not strictly related to this study (e.g. lumen formation, interendothelial junctions...). Again, re-writing to focus on the most relevant points will be necessary.
3. The text is full of abbreviations, which makes reading difficult.
4. The superposition of colors in images is often difficult to understand, and therefore many of them have mainly esthetical value. One could co-present also B&W pictures of the main staining of the complicated images, to help the reader convincingly visualize the important point.
5. Delineation of the CCVs missing on figure 6B.

Referee #3 (General Remarks):

This manuscript brings the power of optical sectioning microscopy approaches to analysis of the early stages of development of the lymphatic vasculature. In-so-doing it reports multiple discoveries including new insight into the role of Ccbe1 in lymphatic development, and the first characterisation of two separate lymphatic vessels that have so far been collectively referred to as the lymph sacs. The manuscript presents very high quality and thorough work in a clear fashion. This work significantly enhances our understanding of early lymphatic development and will likely be considered a "platform" for future studies into molecular and anatomical aspects of the development and function of the lymphatic vasculature. I have no major criticisms of the study and list a few minor points below.

1. Introduction is a little long and reads like part of a thesis chapter - it should be more tightly focussed. Some of the abbreviations are not defined, such as Nrp2 and ISV.
2. Legend to Supplementary Fig. 1: there are some typographical errors, and FW and TL are not defined.
3. Legend to Supplementary Figure 8: it indicates a panel J but I could not find it.

1st Revision - authors' response 01 December 2012

Specific responses to the reviewers' comments

We thank the referees for their helpful and constructive comments, which were valuable in shaping an improved manuscript and which we were able to address in full. In the following, we describe in detail how we have addressed and incorporated the reviewers' suggestions:

Specific responses to the comments of referee #1

We are pleased about referee #1 judging that
The description is rigorous and the figures shown are largely informative.

However the use of Cxcr4 and CCBE1 null mice are not sufficient to describe a solid mechanism explaining the formation of these structures. I suggest to extend the behaviour of pTD and PLLV in Prox1 and VEGFR3 or VEGF-C null mice.
Considering the referee's concerns, we decided to remove the analysis of Cxcr4 from the manuscript in favour of an expanded and more in depth analysis of *Ccbe1* mutant mice. Our decision was based on the following considerations. (1) Inclusion of an extended analysis of both genes would be beyond the scope of a single publication. (2) CCBE1 fulfils an essential function during development of lymphatic vessels in fish, mice and humans. (3) Neither the biological function, nor the molecular defects caused by CCBE1 deficiency are presently understood. (4) Defects caused by CCBE1-deficiency arise during the earliest stages of lymph vessel development and are hence ideally analysed by ultramicroscopy.
We concur with referee #1 and have included the analysis of *Vegfr3* heterozygous and *Vegfc* -/- mice. VEGFR-3 and VEGF-C both fulfil essential functions in early lymphatic development and we therefore found their analysis fitting in the context of this manuscript. (2) We have substantially expanded our analysis of CCBE1 deficiency by including *Vegfc* x *Ccbe1* double heterozygous mice.

Furthermore most of the data shown should be connected to and supported by morphometric analyses of the phenomena described.
We agree - to address this highly relevant point, we have now included a quantitative evaluation of the visual / optical information provided for many figures in the manuscript. In particular, we have included an extensive morphometric analysis of the shape and Prox1 expression of lymphatic progenitors leaving the cardinal vein (Fig 2).

To better support the concept underlined in the II paragraph of page 6 I suggest to show embryos at 9.5 and 10.5 at low magnification as in panel A and B of fig 1 with endomucin and Prox1 both associated with PECAM.
The requested data have been included in Fig.1 C,D and as suppl. Fig.2

Fig 1T. How do the authors record the nuclear shape?
Figure 1 has been extensively rearranged. As pointed out above, the original figure 1T (which reported the nuclear aspect ratio i.e. length max/minor axis) has been replaced by an analysis, which provides precise morphometric data on the shape (Fig.2 D,E ellicticity vs sphericity) and Prox1 expression level (Fig.2 F,G) for the lymphatic progenitors remaining and emerging from the cardinal vein.

Page 7. "The transition was associated with a pronounced...in iLEC (...)." May the authors show that the shaped modified iLEC really increase the expression of VEGFR3, Nrp2 and Prox1? How do the authors support this claim. It is required to show an accurate morphometric analysis. Similar information have to be provided for the description of other gene expression modifications along the text.
We fully concur with the reviewer's notion and now provide quantitative morphometric data e.g. in Fig.2 expression strength is quantified via fluorescence intensity measurements.

Page 7 last paragraph. An elegant experiment to really confirm that LEC number increases from E10 to E10.25 should be done by in vivo BrdU labelling. (similar experiment have been provided for earlier stages, fig s6).
We fully agree and as requested, we have extensively repeated the *in vivo* EdU-labelling experiments and now provide in the new supplementary Fig.8 data on mouse embryos from a wide range of developmental stages (from E9.5, E10.0 and E10.5 to E11.5). Equally surprising and interesting, at no time we did detect a burst in proliferation, suggesting that the massive appearance of iLECs between E10.0 and E10.5 is the direct consequence of rapid emigration from the venous sources rather than increased proliferation.

Fig 2. Which is the panel corresponding to scheme G? I suggest to show sLEC in the wholemount pictures. Alternatively, refer to fig 3C.
We have removed the scheme corresponding to Fig.2 G and in addition corrected our schematic representations such that they now exactly match the wholemount panels.

Fig 3B. Which the rationale for the use of Unc5B? Some of the explanation provided later in the text should be indicated here.
The staining for Unc5B was performed in search for molecular markers that might distinguish the different LEC populations, e.g. sLECs from iLECs forming the PLLV or pTD. An important question is the one for the positional cues that result in the formation of these first lymphatic structures. We follow the suggestion of reviewer #1 and have indicated these issues in the text.

Fig 4E. What is the PECAM positive "ring" near CV?
Due to their high Prox1 expression, arterial vessels appear in a perfectly orthogonal section as an intensely stained ring (red in this case). The vessel in question is an arterial vessel connecting to the subclavian artery. We refer referee #1 to supplemental Fig.3, which provides an overview of the arterial vasculature around at the respective developmental stage.

In Fig 4F the authors describe the interaction between an arterial vessel and CV. This point should be discussed and a possible speculation of this role exploited.
We have included this point in the discussion page 16 and speculated about a possible inductive role of this vessel to the formation of the lymphovenous valves.

Fig 5I. Which is the quantitative basis of the score here reported?
The quantitative assessments in Fig.5 I (now Fig.6 I) are based on antibody stained serial cyrosections that were analysed using a confocal microscope Zeiss LSM780. Importantly, all quantitative statements are based on LEC populations present on the same section. While so some expression changes were very obvious, e.g. complete loss of a protein, others were measured by pixel evaluation of the average pixel luminosities of at least 20 cells. A description of this procedure has now been included in the methods section..

Page 10, first lines. Which is the rationale supporting that valves develop from these Prox1 positive structures? I suggest examining the presence of the contact described in fig 4 in Prox1 and VEGFR3 null mice.
This point raised by reviewer #1 is an important one. While we lack formal proof that these areas will develop into lymphatic valves, the following points provide compelling support for this assumption. We detected exceedingly high Prox1 expression only at the direct contact points between CV and pTD. These contact sites were formed by two layers of endothelial cells, both expressing Prox1. One layer was provided by pTD, the other by the CV. (1) High level Prox1 expression has been reported to be a hallmark of valve induction later during lymphatic vessel maturation (Norrmen *et al.*, 2009) (Sabine *et al.*, 2012). (2) The anatomical position of the described

contact points, corresponds to the position reported in a recent publication, which studied the formation of lymphatic valves (Srinivasan and Oliver, 2011) and corresponds to the contact sides between lymphatic vessels and subclavian veins in the adult (see suppl. Fig.3). In the publication by Srinivasan et al. paired sites of valve formation, exactly like we see them, were shown to be comprised of two layers of endothelium, both expressing Prox1. These areas of the prospective valves are the only sites were Prox1 expression is retained in the CV (Srinivasan *et al.*, 2011). (3) The onset of lymphatic function, which depends on the development of functional valves is reported shortly after the formation of the contact sites between pTD and CV (Planas-Paz *et al.*, 2012).

Fig 6. To facilitate the reader I suggest showing again the schemes of wild type mice.
We agree and gladly include this graphical element also for the newly included *Vegfc* mutants.

Fig 7. Does Cxcr4 ablation modify PPLV condensation?
No it does not. However, due to the extensive revisions requested and the major concern of reviewer #2, we have decided to remove the analysis of the CXCR4 ko from this manuscript and instead (1) we included the analysis of *Vegfr3* heterozygous and *Vegfc* -/- mice. VEGFR-3 and VEGF-C both fulfil essential functions in early lymphatic development and we therefore found their analysis fitting in the context of this manuscript. (2) We have substantially expanded our analysis of CCBE1 deficiency by including *Vegfc* x *Ccbe1* double heterozygous mice.

OTHER POINTS

Fig 1a,B. I suggest to indicate CV not only in the legend but also in the panels.
We indicated the position of the CV in all subsequent and compound panels, however, we find it obstructive when painted into Fig.1 A,B.

Fig 7. The title of figure is confusing
We agree, in any case the figure has been replaced.

Fig 4B "Hear " is probably a mistake
Thank you for pointing this out, we have corrected the mistake.

Fig 1 , panel S. To this referee it is not clear which panel this scheme corresponds.
We have now indicated the relation of this scheme to panel 2B.

Fig 1N,O. There is not correspondence between the text (page 7, III paragraph, line 8) and the embryo stage indicated in the panels. E11 corresponds to Fig 5.
Yes actually there was a mix up in the text, We have corrected the discrepancy between text and Fig.1 N,O which is now Fig. 2A,B.

Specific responses to the comments of Referee #2

Referee #2 (General Remarks):

René Hägerling & Pollmann et al. present an impressive amount of beautiful images depicting early stages of lymphatic vascular development in E9.5-12 mouse embryos. In contrast to recent results of Francois et al., this works shows that lymphatic endothelial cells, arising in the cardinal vein, exit as strings of migratory spindle-shaped cells. ...
... very interesting phenotype of Ccbe1 deficient mice is described only briefly. More detailed comments are outlined below:

MAJOR REMARKS

1. The manuscript should be re-written and shortened to emphasize two essential point of the manuscript, i.e. the new model for emergence of lymphatic vasculature, and better characterization of Ccbe1 knockout mice. If such possibility exists, I would recommend a short report format.

We appreciate the overwhelmingly positive comments of reviewer #2 and have very seriously considered the reviewer's concerns. Indeed, following the suggestions of reviewer #2 we have rewritten or revised the entire manuscript. As suggested by the reviewer, we have deleted the analysis of Cxcr4, substantially expanded the analysis of the *Ccbe1 -/-* mice and include analysis of *Vegfr3* heterozygous and *Vegfc* -/- mice. We feel that this amount of data can not be sufficiently represented in a short format article.

2. The number of images should be reduced by approximately half, only essential high magnification images clearly illustrating the point should be shown. In my view, almost all figures require this clean-up. As example, in figure 1N, O only one picture, that illustrates changes in nuclear shape should be retained. In supplementary figure 8, only image per developmental stage showing the expression domain of Ccbe1 should be shown, which will make three images instead of current nine, etc.
We have very carefully reassessed the value and necessity of all figures in the manuscript and have for instance, in agreement with the referee's suggestion deleted the panel corresponding to the original Fig. 1M. Also we have removed a substantial amount of panels and reduced, as requested, the Ccbe1 lacZ data (now suppl. Fig.10) to three panels. We do, however, strongly feel that a further reduction of the number of images to half the original panels, would remove important and indispensable information from the manuscript. In particular, we feel very strongly about the low magnification overview images, which with unprecedented detail allow the reader to relate lymphangiogenesis to the entire developing embryo, which we feel is of tremendous value, as shown e.g. in suppl. figure 3 which allows the identification of a particular arterial structure, which would be close to impossible from a serial sectioning approach.

3. The final figure should contain the comparison of the previous and new scheme that authors propose for formation of early lymphatic vasculature.
Given the large amount of graphic material that had to be accommodated in this manuscript, we would like to focus on primary data here and postpone the generation of a scheme for later publications.

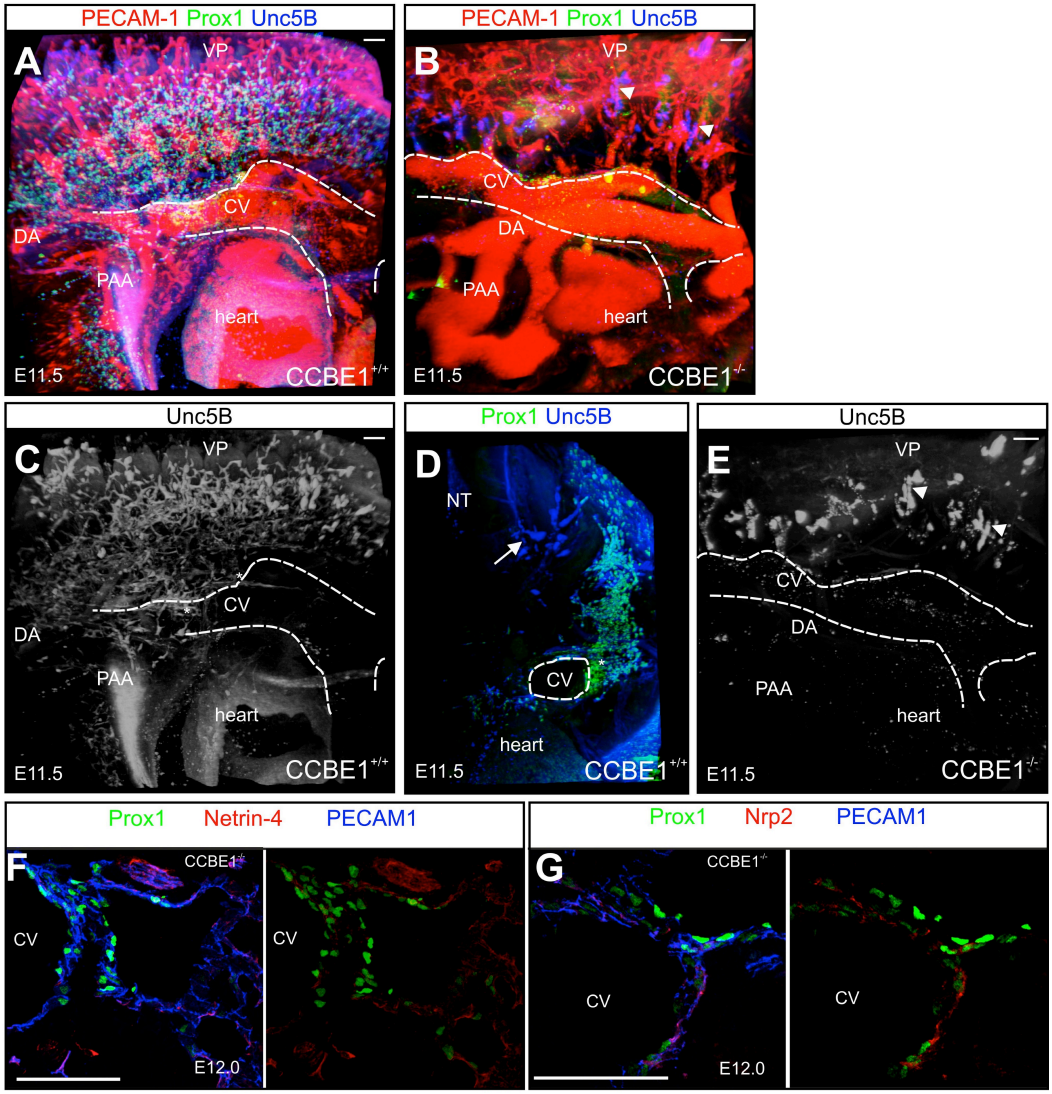
4. Description of expression of lymphatic markers should be shortened or re-written. In the present manuscript, this description looks like a patchwork, and one wonders why expression of other molecules, important for vascular development, for example Vegfr2 (spouting), ephrinb2 (Vegfr2/3 signalling), integrin alpha9 (vessel maturation and LECs migration), integrin beta1 (lumen formation) was not studied?
Based on the referee's comments, we have re-written the description of the lymphatic markers. We welcome the suggestions and have included the additional marker proteins VEGFR-2, integrin b1 and a9 (suppl. Fig.6). We have extensively attempted to stain integrin a9, however, using a protocol that reproducibly detected a9 staining during the last third of mouse development, we were not able to visualize a9 during the earliest stages of lymph vessel development.

5. *Down regulation of Vegfr-3 in blood vessels, emergence of podoplanin expression only at E11 and lack of Lyve-1 expression in early dermal lymphatic vessels are not entirely new findings, corresponding papers (Kaipainen et al., 1995, Schacht et al., 2003, Norrmen et al, 2009) should be cited. High Nrp2 and low Lyve1 in migrating LECs were also described by Francois et al., 2011.*
We apologize if the reader might have been misled to assume that these findings were novel. As pointed out by referee #2 they have been reported previously and we have made this more clear and have included the relevant references on page 5 and 9 to clarify this fact.

6. Description of Ccbe1 phenotype is confusing. Do the authors suggest that in the absence of Ccbe1 there is ectopic trans-differentiation of LECs in ISVs and other venous endothelium? How does it fit with the statement that Ccbe1 is necessary for the maintenance of Prox1 expression? What is the mechanisms for increased Vegfr-3 expression? Was the expression of Nrp2, netrin-4 and Unc5b modified in Ccbe1 ko embryos, which may in part explain sprouting phenotypes? In any case, this part needs to be better structured and presented more clearly
As pointed out in this reply above, we have addressed these issues by a significantly more in depth analysis of CCBE-1-deficient embryos. To briefly address the reviewer's questions: Our analysis suggested that Prox1 positive cells arise in the CV and ISVs as they do in wild type littermates. The fact that the Prox1-expression domain appeared expanded may simply reflect the incapacity of these cells to leave the CV. Their differentiation state in the CV appeared to be still plastic and they failed

to maintain Prox1 expression over the next 24hrs. The requested data on Nrp2, netrin-4 and Unc5b have been provided for the reviewer (see suppl. Fig. for reviewer #2). Expression of Unc5b was lost from the CV, but also from Prox1+ cells in Ccbe -/- embryos. Nrp2 was only detectable on the CV, while Prox1+ cells were negative. Netrin-4 finally was only found very weak on the CV. While this pattern is difficult to interpret and we certainly still miss the molecular mechanisms underlying the phenomena observed in *Ccbe1 -/-* mice, we would like to point out, that our description is presently the most detailed analysis of CCBE-1 action during lymph vessel development. It has revealed previously unappreciated details e.g. the failure of Prox-1 positive cells to leave the CV due to their inability to undergo an EMT type of transformation in the absence of CCBE-1. In this context, we would like to point out, that several groups are intensely, but so far unsuccessfully, searching for a receptor for CCBE1, which will certainly be a key element in the understanding of this molecule.

Supplementary Figure for reviewer #2 (Hägerling & Pollmann et al.)



Unc5B is not expressed in the CV of CCBE1-deficient embryos at E11.5.
(**A-E**) Sagittal and (**D**) transversal optical sections of *Ccbe1-/-* (**B,E**) and *Ccbe1+/+* (**A,C,D**) embryos at E11.5, wholemount-immunostained for the indicated proteins. (**F,G**) Immunostainings of histological cryosections of *Ccbe1-/-* Embryos at E12.0. Colors as noted in the labeling above each panel. Long-hatched lines denote the position of the CV. Cranial, left; caudal, right.
(**B,E**) In *Ccbe1-/-* embryos Unc5B staining was not detected in the CV or the aberrant sprouts extending from it. The less intense signal (arrowheads) mainly represents Unc5B positive structures in the perineural vascular plexus, which are also detectable in control embryos (G, arrow).
(**F**) Netrin-4 is weakly expressed on Prox1+ cells of the CV.
(**G**) Prox1+ cells of the CV are Nrp2 positive, while iLECs are negative.
Scale bars = 100 µm.

7. CXCR4 story does not really fit with the other parts of the study, which focuses on the very early steps of lymphatic vascular development. At E14.5 in the skin the lymphatic developing vasculature is still growing as an immature primary plexus and the differentiation into capillary and collecting vessels is therefore difficult to visualize at that early stage. Given a rather superficial characterization of Cxcr4 knockout phenotype, this part can be removed without affecting main message (and perhaps published separately after more detailed analysis).
We fully agree, the analysis of CXCR4 has been removed from the manuscript.

Minor remarks

1. The paragraph at the beginning of Results section describing microscopy is very technical, I suggest to shift it to the Materials and Methods.
We object, use of ultramicroscopy has been central to the entire study and we find the technical details provided in this first paragraph are easy to grasp but also indispensible for the reader to correctly understand and judge the data presented. In addition, we have revised and shortened this paragraph.

2. Some paragraphs of the discussion are redundant with the results part and some others are not strictly related to this study (e.g. lumen formation, interendothelial junctions...). Again, re-writing to focus on the most relevant points will be necessary.
Agree, during the revision of the manuscript, we have also rewritten the discussion and removed redundant parts.

3. The text is full of abbreviations, which makes reading difficult.
While we see the referee's point, we don't agree entirely. All abbreviations are carefully introduced, the corresponding structures are explained in detail and identical abbreviations are used throughout in the text and graphic material. We feel that this makes the manuscript more accessible to the reader than numerous repetitions of the complex full terms.

4. The superposition of colours in images is often difficult to understand, and therefore many of them have mainly esthetical value. One could co-present also B&W pictures of the main staining of the complicated images, to help the reader convincingly visualize the important point.
Following the reviewers suggestion, we have converted a number of colour panels to B&W, to facilitate optical access. Also single colour plates of complex multicolour stainings were included (e.g. suppl. Fig. 2 and 9). However, we still feel that multicolour depiction is an excellent form of representing the relationship of different developing organs or tissue types. We fully appreciate the complexity of the graphical depictions and have therefore also included video material with our revision.

5. Delineation of the CCVs missing on figure 6B.
The missing element has been introduced into the Fig, now Fig. 7B

Referee #3 (General Remarks):

This manuscript brings the power of optical sectioning microscopy approaches to analysis of the early stages of development of the lymphatic vasculature. In-so-doing it reports multiple discoveries including new insight into the role of Ccbe1 in lymphatic development, and the first characterisation of two separate lymphatic vessels that have so far been collectively referred to as the lymph sacs. The manuscript presents very high quality and thorough work in a clear fashion. This work significantly enhances our understanding of early lymphatic development and will likely be considered a "platform" for future studies into molecular and anatomical aspects of the development and function of the lymphatic vasculature. I have no major criticisms of the study and list a few minor points below.

1. Introduction is a little long and reads like part of a thesis chapter - it should be more tightly focussed. Some of the abbreviations are not defined, such as Nrp2 and ISV.
We thank referee # 3 for his very positive criticism and are happy to report that we have revised and shorted the introductory chapter and also included the missing abbreviations.

2. Legend to Supplementary Fig. 1: there are some typographical errors, and FW and TL are not defined.
We appreciate this valuable comment and have corrected legend and the missing definitions.

3. Legend to Supplementary Figure 8: it indicates a panel J but I could not find it.
Supplementary Fig.8 now supplementary Fig.10 has been completely reworked and correct labelling has been assured.

REFERENCES

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